Biochimica et Biophysica Acta 1778 (2008) 1501–1507

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Importance of the phosphocholine linkage on sphingomyelin molecular properties and interactions with cholesterol; a study with phosphate oxygen modified sphingomyelin-analogues

Anders Björkbom [a,*], Tetsuya Yamamoto [b], Satoshi Kaji [b], Shuji Harada [b], Shigeo Katsumura [b], J. Peter Slotte [a]

[a] Department of Biochemistry and Pharmacy, Åbo Akademi University, Tykistökatu 6 A, FIN-20520 Turku, Finland
[b] School of Science and Technology, Kwansei Gakuin University, 2-1 Gakuen, Sanda City, Hyogo 669-1337, Japan

ARTICLE INFO

Article history:
Received 21 January 2008
Received in revised form 3 March 2008
Accepted 3 March 2008
Available online 18 March 2008

Keywords:
Sphingomyelin interactions
Fluorescence quenching
Anisotropy
Phosphocholine linkage
Membrane–water interface
Differential scanning calorimetry

ABSTRACT

We have characterized the molecular properties and membrane behavior of synthetically modified sphingomyelin analogues, modified on the oxygen connecting the phosphocholine group to the ceramide backbone. The oxygen was replaced with an S-atom (S-PSM), an NH-group (NH-PSM) or a CH_2-group (CH_2-PSM). Diphenylhexatriene and Laurdan anisotropy experiments showed that an S-linkage increased and NH- and CH_2-linkages decreased the stability of PSM-analogue bilayer membranes as compared to PSM. When the polarity of the interface was probed using Laurdan, S-PSM appeared to have a lower polarity as compared to PSM whereas NH-PSM and CH_2-PSM had higher polarities of their respective interfaces. Fluorescence quenching-studies with cholestatrienol showed that all compounds formed SM/cholesterol-rich domains. The S-PSM/cholesterol and PSM/cholesterol domains displayed a similar thermostability, whereas NH-PSM/cholesterol and CH_2-PSM/cholesterol domains were less thermostable. DSC on vesicles containing the PSM-analogues showed a more complex melting behavior as compared to PSM, whereas equimolar mixtures of the PSM-analogues and PSM showed almost ideal mixing with PSM for NH- and S-PSM. Our data show that the properties of the bond linking the phosphocholine head group to the 1-hydroxyl on the ceramide molecule is important for the stability of SM/SM and SM/cholesterol interactions.

1. Introduction

Interactions between sphingolipids in bilayers occur both between head groups at the interface of the bilayer, and between the *N*-linked acyl chains and the sphingosine backbones in the hydrophobic part of the bilayer. For sphingomyelins (SMs) it has been suggested, based on NMR spectroscopy, that there would exist an intramolecular hydrogen bond between the hydroxyl group in the sphingosine backbone and the proximal esteric oxygen (the oxygen connecting the phosphocholine head group to the ceramide backbone) in the phosphocholine head group [1,2]. This observation is supported by molecular dynamics simulations in PSM and PSM/POPC/cholesterol bilayers above the T_m of PSM. The molecular dynamics simulations showed the existence of intramolecular hydrogen bonds between the hydroxyl group and the proximal esteric oxygen of SM [3,4]. It has further been suggested that the amide group of the sphingosine backbone acts as a donor in SM/SM and SM/water intermolecular hydrogen bonding [2,3]. We have previously investigated the effect of the *trans*-double bond in the sphingosine backbone upon SM intermolecular interactions and compared to D-*erythro*-*N*-palmitoyl-sphingomyelin (PSM), D-*erythro*-*N*-palmitoyl-dihydrosphingomyelin (DHSM) forms more stable bilayer membranes [5–7] In agreement with NMR studies, these observations could be explained by closer contact between the DHSM molecules, the closer contact giving rise to stronger intermolecular hydrogen bonding and weaker intramolecular hydrogen bonding [2].

The interactions between cholesterol and SMs that take place during the formation of ordered domains are complex and of varying nature (for reviews see [8,9]). The importance of intermolecular hydrogen bonding between the hydroxyl group in cholesterol and the amide group in SM is still unclear. The amide group in SM has been suggested to be important in interactions with cholesterol in SUVs and monolayers [10]. Further, infrared and spin label electron spin resonance spectroscopy studies on egg-SM/cholesterol mixtures propose the existence of

Abbreviations: 7SLPC, 1-palmitoyl-2-stearoyl-(7-DOXYL)-*sn*-glycero-3-phosphocholine; CH_2-PSM, PSM with its proximal esteric oxygen replaced with a CH_2-group; CTL, cholesta-5,7,9(11)-trien-3-beta-ol; DHSM, D-*erythro*-*N*-palmitoyl-dihydrosphingomyelin; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSC, differential scanning calorimetry; GP, generalized polarization; Laurdan, 6-lauroyl-2-(*N*,*N*-dimethylamino)naphthalene; l_d, liquid disordered-phase; l_o, liquid ordered-phase; NH-PSM, PSM with its proximal esteric oxygen replaced with an NH-group; PCPE, *N*-palmitoyl ceramide phosphoethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PSM, D-*erythro*-*N*-palmitoyl-sphingomyelin; SM, sphingomyelin; S-PSM, PSM with its proximal esteric oxygen replaced with a S-atom; T_m, mid temperature of the transition between gel and l_d-phase

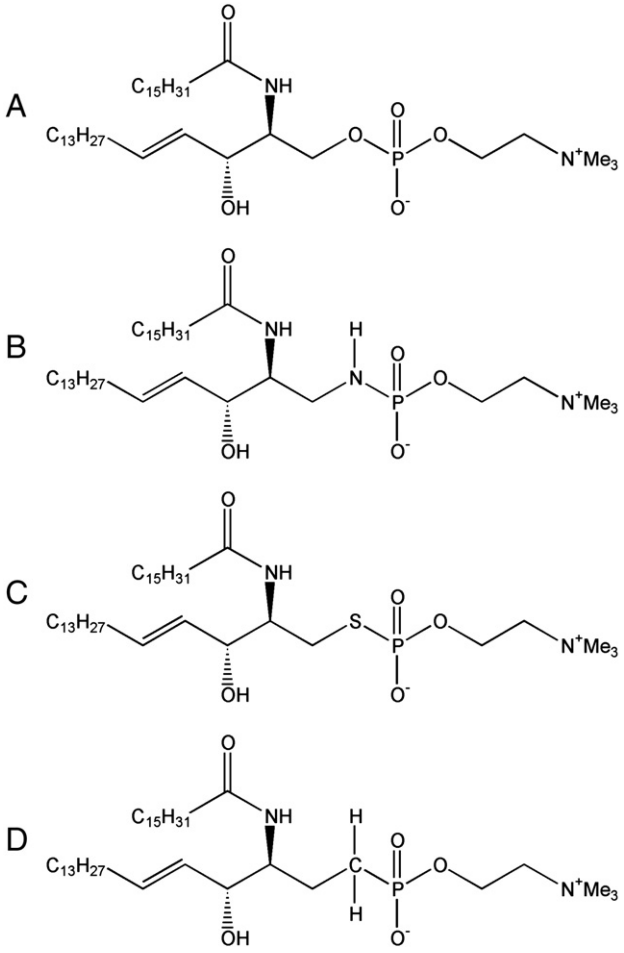
* Corresponding author. Tel.: +358 400822164; fax: +358 2 215 4745.
E-mail address: abjorkbo@abo.fi (A. Björkbom).

0005-2736/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2008.03.005

hydrogen bonds between the amide group in SM and the hydroxyl group of cholesterol [11]. However solid state NMR studies examining hydrogen bonding between bovine brain-SM and cholesterol found only small indications of hydrogen bonding at high cholesterol levels and no hydrogen bonding at low cholesterol levels [12]. Computational simulations, on the other hand, support the existence of hydrogen bonds between cholesterol and SM in binary mixtures [13,14]. However, also other interfacial interactions such as charge-pairing and water bridging are likely to be important for the preferential SM/cholesterol interactions [4].

The size of the head group in phospholipids has been shown to be important in the interactions with cholesterol [15,16]. In monolayer experiments with *N*-palmitoyl ceramide phosphoethanolamine (PCPE) it was shown that PCPEs experienced tighter packing of the acyl chains as compared to PSM. The smaller head group and tighter packing of PCPE molecules in bilayers affects the interactions with cholesterol and fluorescence quenching experiments examining the domain forming properties of PCPE showed that PCPE was in fact not able to form cholesterol-rich domains [16]. PCPE, lacking three methyl groups, has a smaller head group than PSM which might affect the shielding and the bilayer positioning of the cholesterol molecule and thus affect the interactions between cholesterol and PCPE in the hydrophobic part of the bilayer [16,17]. Molecular dynamics simulations have shown that cholesterol is located deeper in the bilayer in SM/cholesterol than in PC/cholesterol bilayers [14]. Deeper anchoring of cholesterol in SM bilayers is suggested to be stabilized by hydrogen bonds between SM and cholesterol and also to induce stronger effects on the acyl chains in the bilayer [14]. Computational simulations on complex lipid mixtures with cholesterol, PSM and POPC have demonstrated that the orientation of the phosphocholine head group of PSM is affected by the presence of cholesterol. The head group is shifted towards the bilayer (the angle to the outward bilayer normal is increased) when PSM comes in contact with cholesterol. This might be a consequence of the free space above the small hydroxyl group of cholesterol or charge-pairing between the choline nitrogen and the oxygen in cholesterol [4]. Computational simulations have also shown an increase in SM intramolecular hydrogen bonding, between the hydroxyl group and the phosphate oxygens of SM, as SM comes in contact with cholesterol [4,13].

In this study we investigated how structural modifications of SMs in the head group region affect properties of SMs at the interface and in the inner, hydrophobic part of the bilayer. The synthetic proximal esteric oxygen-modified PSM-analogues examined in this study were originally synthesized as sphingomyelinase inhibitors [18–20]. The PSM-analogues were modified on the oxygen connecting the phosphocholine head group to the ceramide backbone replacing it with an NH-group, S-atom or CH_2-group, yielding NH-PSM, S-PSM and CH_2-PSM. It has been shown that both NH-PSM and CH_2-PSM inhibited the activity of SMase (*B. cereus*) significantly, whereas S-PSM only showed weak inhibition of SMase activity [18–20]. Scheme 1 shows the structures for PSM and the PSM-analogues. The replacement of the proximal esteric oxygen is likely to affect the properties of the PSM-analogues and the aim of this study was to examine how the modifications affect the SM properties and interactions with cholesterol in multilamellar bilayer vesicles. An abstract of this study has previously been presented at an international meeting [21].



Scheme 1. Structures of PSM and the PSM-analogues. For the PSM-analogues the oxygen connecting the phosphocholine head group to the ceramide backbone (proximal esteric oxygen) in PSM (A) has been replaced with an NH-group (B), an S-atom (C) or a CH_2-group (D).

2. Materials and methods

2.1. Materials

NH-PSM, S-PSM and CH_2-PSM were obtained from professor Katsumura at the Kwansei Gakuin University in Japan [18–20]. The PSM-analogues were purified using reverse-phase HPLC (Supelco Discovery C-18 column, 25 cm×21.2 mm, 5 μm particle size) with 100% methanol (Rathburn Chemicals Ltd, Walkerburn, Scotland) and acetonitrile/methanol (Rathburn Chemicals Ltd, Walkerburn, Scotland) (50:50, vol.%) as the mobile phase. PSM was purified from egg-SM (Avanti Polar-Lipids inc., Alabaster, AL, USA) through reverse-phase HPLC (Supelco Discovery C-18 column, 25 cm×21.2 mm, 5 μm particle size) using 100% methanol (Rathburn Chemicals Ltd) and acetonitrile/methanol (Rathburn Chemicals Ltd) (50:50, vol.%) as the mobile phase. The purity and identity of PSM and the PSM-analogues was checked on a Micromass Quattro II mass spectrometer (Manchester, UK). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids and cholesterol was obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Stock solutions of the lipids were dissolved in hexane/2-propanol (J.T. Baker, Mallinckrodt Baker, Deventer, Holland) (3:2, vol:vol) and stored at −20 °C in the dark. Before use the stock solutions were heated to ambient temperature.

Cholesta-5,7,9(11)-trien-3-beta-ol (CTL) was synthesized using the method described in [22]. The synthesis products were purified using reverse-phase HPLC (Supelco Discovery C-18 column, 25 cm×21.2 mm, 5 μm particle size) with 95% methanol (Rathburn Chemicals Ltd) and 5% hexane (J.T. Baker) as the mobile phase. (7-Doxyl)-stearic acid (TCI Europe N.V., Belgium) and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Avanti Polar-Lipids) were used in the synthesis of 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine (7SLPC) according to [23]. The synthesis products were purified using reverse-phase HPLC (Supelco Discovery C-18 column, 25 cm×21.2 mm, 5 μm particle size) with 100% methanol (Rathburn Chemicals Ltd) as the mobile phase. The purity and identity of 7SLPC was checked on a Micromass Quattro II mass spectrometer. CTL and 7SLPC were stored in the dark at −87 °C in an argon environment. Before use the lipids were dissolved in argon purged ethanol (CTL) or hexane/2-propanol, (2:3, vol:vol) (7SLPC) and used within 3 days. 1,6-Diphenyl-3,5-hexatriene (DPH) and 6-lauroyl-2-(*N*,*N*-dimethylamino)naphthalene (Laurdan) were purchased from Molecular Probes (Leiden, The Netherlands). The H_2O used in all experiments was purified with a Millipore UF Plus purification system, giving a product with an 18.2 MΩ cm resistivity called mQ-H_2O. All solvents used were purged with argon and all fluorescent probes were protected from light to avoid oxidation.

2.2. Preparation of multilamellar vesicles

Vesicles used in the DPH anisotropy and Laurdan experiments were prepared to a final lipid concentration of 25 μM and vesicles used in the fluorescence quenching

experiments were prepared to a final lipid concentration of 50 μM. The varying lipid mixtures were prepared from lipid bulk-solutions, dried under nitrogen at 40 °C and kept in vacuum at 22 °C for at least 30 min. The dry lipid mixtures were then dispersed in-argon purged mQ-H_2O and heated to a temperature exceeding the T_m (mid temperature of the transition from gel to liquid-crystalline state) of the lipids used (approximately 60 °C), for 20 min. The heated samples were vortexed and probe sonicated for 2 min using a Branson probe sonifier W-450 (25% duty cycle, power output 10 W, Branson ultrasonics, Danbury CT, USA) yielding multilamellar and multisize liposomes. For pure PSM vesicles the mean diameter was approximately 200 nm and the size distribution was rather large. The size distribution and mean diameter were determined using a Malvern Zetasizer Nano-S particle analyzer (Malvern instruments Ltd, Worcestershire, UK) at an angle of 173°.

For the steady-state fluorescence anisotropy measurements of DPH the samples consisted of PSM, NH-PSM, S-PSM or CH_2-PSM with 1 mol% DPH. Cholesterol was added to the lipid mixtures replacing 20 mol% and 30 mol% of the respective lipids. For the steady-state fluorescence anisotropy measurements of Laurdan the samples consisted of PSM, NH-PSM, S-PSM or CH_2-PSM with 1 mol% Laurdan. In the fluorescence quenching experiments the *F*-samples consisted of POPC/7SLPC/varying lipid/cholesterol (30:30:30:10, mol%) and the F_0-samples consisted of POPC/varying lipid/cholesterol (60:30:10). The varying lipid was PSM, NH-PSM, S-PSM or CH_2-PSM and the samples were probed with 1 mol% of CTL, replacing cholesterol. The samples for differential scanning calorimetry (DSC) experiments were prepared to final lipid concentration of 0.7 mM for pure and mixed bilayer vesicles. The pure bilayer vesicles contained PSM, NH-PSM, S-PSM or CH_2-PSM and the samples with mixed compositions consisted of NH-PSM, S-PSM or CH_2-PSM together with 50 mol% PSM. The lipid mixtures were prepared from lipid bulk-solutions, dried under nitrogen at 40 °C and kept in vacuum at 22 °C for at least 30 min. The dry lipid mixtures were then dispersed in-argon purged mQ-H_2O and heated to a temperature exceeding the T_m of the lipids used (approximately 60 °C), for 20 min. The vesicles were prepared through brief vortexing (10 s) followed by bath sonication (1 min) on Branson 2510E-MT ultrasonic cleaner (Branson Ultrasonics, Danbury, CT, USA) at temperatures exceeding the T_m (approximately 60 °C) for the respective lipids.

2.3. Steady-state fluorescence anisotropy measurements

Steady-state fluorescence anisotropy measurements were performed on a PTI QuantaMaster-1 spectrofluorimeter (Photon Technology International, Birmingham, NJ, USA) working in the T-format. The pure multilamellar bilayer vesicles were prepared as described earlier and the DPH and Laurdan concentrations were 1 mol%. The DPH anisotropy was also measured in vesicles containing 20 mol% and 30 mol% cholesterol replacing the respective PSM-analogue. For DPH and Laurdan anisotropy measurements the emission and excitation slits were set to 5 nm. The temperature was controlled by a Peltier element with a temperature probe submerged in the samples. The samples were heated with 5 °C/min and constantly stirred at 260 rpm. DPH was excited at 360 nm and the emission was detected at 430 nm and Laurdan was excited at 360 and the emission was detected at 450 nm. The T_ms from the anisotropy experiment were determined visually. The anisotropy was calculated as described in [24].

2.4. Laurdan emission measurements

The multilamellar bilayer vesicles were prepared as described above with 1 mol% of Laurdan. Laurdan was excited at 360 nm and the emission spectra were detected at indicated temperatures between 390 and 550 nm on a PTI QuantaMaster-1 spectrofluorimeter working in the T-format. The emission and excitations slits were set to 5 nm and the temperature was controlled by a Peltier element with a temperature probe submerged in the samples. The samples were constantly stirred at 260 rpm. The calculation of the generalized polarization (GP) is described in Eq. (1).

$$GP = (I_{435} - I_{505})/(I_{435} + I_{505}) \quad (1)$$

I_{435} is the fluorescence intensity at 435 nm and I_{505} is the fluorescence intensity at 505 nm. The GP-value indicates the spectral shift in fluorescence intensity as the environment around Laurdan is changed [25].

2.5. Fluorescence quenching measurements

Fluorescence quenching experiments have been thoroughly described in [26,27] and the preparation of *F*- and F_0-samples has been described above. Fluorescence quenching experiments were performed on a PTI QuantaMaster-1 spectrofluorimeter and the temperature was controlled by a Peltier element with a temperature probe submerged in the samples. The samples were heated with 5 °C/min and constantly stirred at 260 rpm.The emission and excitations slits were set to 5 nm and CTL was excited at 324 and the emission intensity was measured at 374 nm. The temperature at complete melting of cholesterol-rich ordered domains was determined visually.

2.6. Differential scanning calorimetry measurements

The vesicles and their compositions were prepared as described above. The DSC experiments were performed on a Calorimetry Sciences Corporations Nano II DSC (Lindon, UT, USA). The samples were analyzed between 15 °C and 75 °C at least three times at 0.5 °C/min. The analysis of raw data was performed using Cpcalc 2.1 (Applied Thermodynamics) and Microcal Origin 6.0 (Microcal Software Inc., Northampton, MA, USA).

3. Results

3.1. Steady-state anisotropy of DPH and Laurdan in pure multilamellar bilayer vesicles and vesicles containing 20 mol% and 30 mol% cholesterol

The effect of substituting the proximal esteric oxygen of SM with a NH-, S- or CH_2-group, upon the order of the acyl chains was examined using steady-state anisotropy of DPH in pure multilamellar bilayer vesicles containing PSM and the PSM-analogues. DPH has been shown to localize into the hydrophobic acyl chain-region of the bilayer and thus the orientation of DPH will be sensitive to the order of the acyl chains [28]. The interfacial properties of SMs seemed to affect the order of the acyl chains both in the gel phase and in the liquid disordered-phase (l_d-phase). Fig. 1A shows the DPH anisotropy for pure multilamellar bilayer vesicles containing PSM, NH-PSM, S-PSM or CH_2-PSM as a function of temperature. The transition from gel phase to the l_d-phase is seen as a sharp reduction of the anisotropy. The T_m for PSM (41.5 °C) is in good agreement with earlier data measured by DPH anisotropy and DSC [5]. Compared to PSM, NH-PSM and CH_2-PSM had a somewhat lower T_m whereas S-PSM had a clearly higher T_m. The anisotropy in the gel phase was approximately the

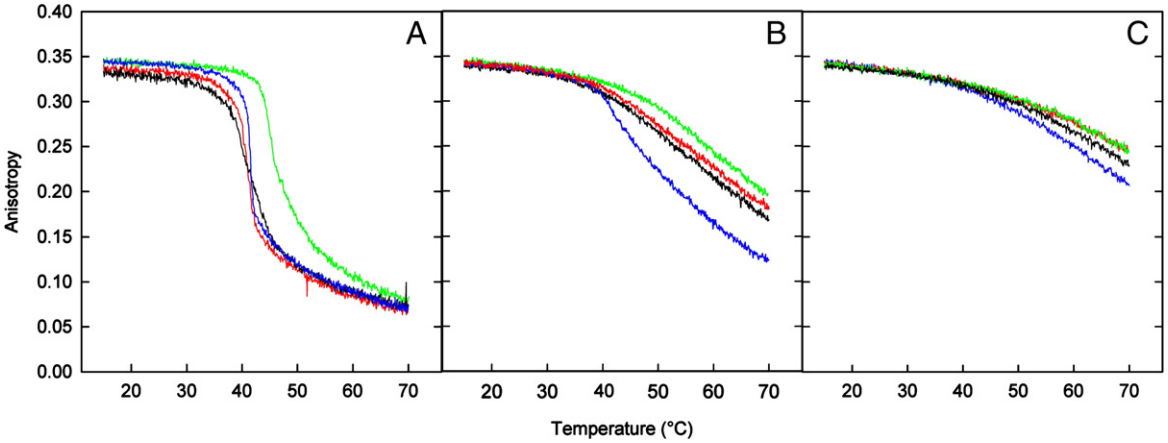


Fig. 1. Steady-state anisotropy of DPH in multilamellar bilayer vesicles containing PSM-analogues and cholesterol. The vesicles contained PSM (blue), NH-PSM (red), S-PSM (green) and CH_2-PSM (black) together with 0 mol% (A), 20 mol% (B) and 30 mol% (C) cholesterol. The samples contained 1 mol% DPH and the lipid concentration was 25 μM. The graphs are representatives of several independent experiments.

same for PSM and S-PSM whereas NH-PSM and CH_2-PSM had a somewhat lower anisotropy. In the l_d-phase the anisotropy for S-PSM was higher than for the other three compounds, having approximately the same anisotropy.

Laurdan is localized closer to the interface of the bilayer as compared to DPH and the steady-state anisotropy of Laurdan showed the order of the membrane closer to the membrane interface as compared to DPH [28–30]. In Fig. 2 the Laurdan anisotropy for pure multilamellar bilayer vesicles containing PSM and the PSM-analogues is shown. Compared to PSM the PSM-analogues all had lower anisotropies in the gel phase. The anisotropy of Laurdan was also lower for PSM and all PSM-analogues as compared to the anisotropy of DPH in the gel phase. The anisotropy of Laurdan was clearly affected by the modifications in the PSM-analogues, since the order in one component bilayers containing the PSM-analogues was lower as compared to corresponding bilayers containing PSM. The T_m for PSM and the PSM-analogues are 1–3 °C lower as compared to the T_ms measured with DPH anisotropy. The differences in T_m between PSM and the PSM-analogues according to the anisotropy of Laurdan were in good agreement with the DPH anisotropy results, S-PSM > PSM > NH-PSM = CH_2-PSM.

To examine how the interaction between the PSM-analogues and cholesterol affected acyl chain order in the bilayers, DPH anisotropy of vesicles containing the PSM-analogues and cholesterol was measured. Above T_m, 20 mol% (Fig. 1B) of cholesterol increased the order in bilayers containing PSM and the PSM-analogues as compared to vesicles without cholesterol (Fig. 1A). 30 mol% (Fig. 1C) of cholesterol further increased the order in the bilayer. The results show that cholesterol had an ordering effect on bilayers containing the PSM-analogues, the increase in membrane order above T_m was in fact larger for the PSM-analogues as compared to PSM. The results suggest that the O-linkage in PSM is important but not essential for interactions with cholesterol.

3.2. Generalized polarization of Laurdan

The emission spectrum of Laurdan is sensitive to the polarity of its environment and an increased polarity at the interface will give a red shift of the emission spectrum. By calculating the GP-values for Laurdan at different temperatures, differences in the hydration of the interface can be estimated, assuming that the membrane depth of Laurdan is not affected by the experimental conditions [25,30,31]. Using Laurdan we probed pure multilamellar bilayer vesicles containing PSM and the PSM-analogues for differences in interfacial polarity,

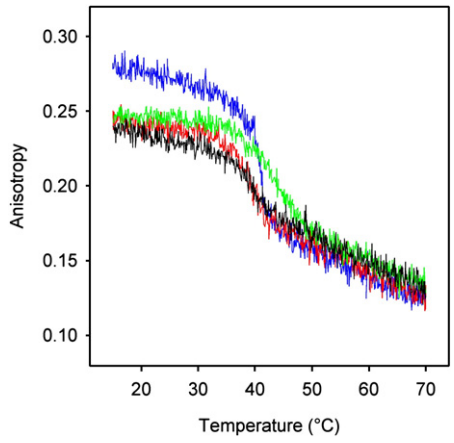


Fig. 2. Steady-state anisotropy of Laurdan in multilamellar bilayer vesicles. The vesicles contained PSM (blue), NH-PSM (red), S-PSM (green) and CH_2-PSM (black). The samples contained 1 mol% Laurdan and the lipid concentration was 25 μM. The graphs are representatives of several independent experiments.

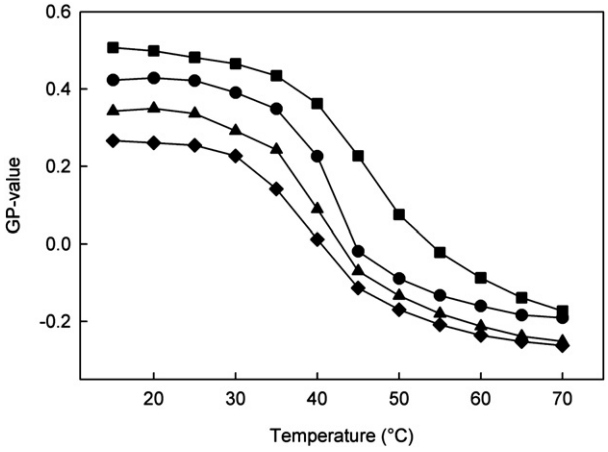


Fig. 3. Generalized polarization (GP) of Laurdan in multilamellar bilayer vesicles. The vesicles contained PSM (circle), NH-PSM (triangle), S-PSM (square) and CH_2-PSM (diamond) and the samples were probed with 1 mol% Laurdan. The lipid concentration was 25 μM and the graphs are representatives of several independent experiments.

Fig. 3 shows the GP-values as a function of temperature. Increased polarity of the interface leads to excited-state dipole relaxation of Laurdan and a red shift of the emission spectra (this results in lower GP-values [30]). Below T_m, S-PSM had the most blue-shifted emission spectra (high GP-values) indicating the lowest hydration of the interface. NH-PSM and CH_2-PSM had more red shifted emission spectra as compared to PSM indicating higher hydrations of the interfaces in the gel phase. In response to the molecular rearrangements occurring during the transition from gel- to l_d-phase, the hydration of the interface increased for all compounds examined. At T_m + 5 °C (T_m from DSC data) S-PSM bilayers had the lowest hydration of the interface and NH-PSM and CH_2-PSM containing bilayers had higher hydrations of the interfaces as compared to bilayers with PSM.

3.3. DSC measurements of pure and mixed bilayer vesicles containing equimolar amounts of PSM-analogues and PSM

DSC was used to study the thermotropic properties of pure PSM-analogues and equimolar binary mixtures of PSM-analogues and PSM. Heating thermograms of the experiments are shown in Fig. 4. In agreement with the anisotropy data, S-PSM (4C, 43.6 °C, ΔH = 7.3 kcal/mol) had a higher T_m than PSM (4A, 41.1 °C, ΔH = 9.8 kcal/mol) whereas NH-PSM (4B, 39.9 °C, ΔH = 6.9 kcal/mol) and CH_2-PSM (4D, 39.0 °C, ΔH = 8.4 kcal/mol) had lower T_ms as compared to PSM. The cooperativity of the main transition was lower for the PSM-analogues as compared to PSM and the melting behavior of the PSM-analogues was more complex as compared to PSM. The enthalpy of the main transition was lower for all PSM-analogues, indicating weaker interactions between the PSM-analogues as compared to interactions between the PSMs (the main transition enthalpy for CH_2-PSM was calculated for the entire multi-state transition). The temperature for the pretransition of NH-PSM (30.4 °C) was similar to that of PSM (29.9 °C) whereas S-PSM did not appear to have a pretransition. CH_2-PSM had a possible pretransition at 35.5 °C, but since it occurred close to the main transition the nature of the low-energy transition remains unknown.

Fig. 4E–H show heating thermograms of equimolar binary mixtures of PSM and each of the PSM-analogues. The results show that NH-PSM (40.7 °C, ΔH = 8.5 kcal/mol) and S-PSM (42.3 °C, ΔH = 7.1 kcal/mol) mixed almost ideally with PSM and that the pretransitions of NH-PSM and PSM were almost completely abolished. The binary mixture of CH_2-PSM (40.0 °C, ΔH = 8.5 kcal/mol) and PSM experienced non-ideal mixing (deconvolution of the main transition showed multiple peaks, data not shown) and the pretransition of the

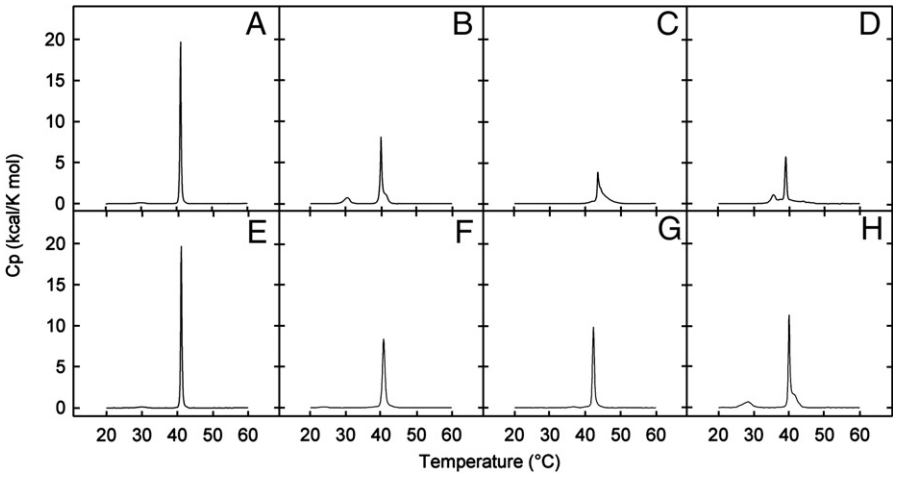


Fig. 4. DSC heating thermograms for PSM-analogues and 1:1 mixtures of PSM-analogues and PSM. The samples for the DSC experiments were prepared to final lipid concentration of 0.7 mM in mQ-H_2O containing PSM (A and E), NH-PSM (B), S-PSM (C), CH_2-PSM (D), NH-PSM/PSM (F), S-PSM/PSM (G) and CH_2-PSM/PSM (H). The samples were analyzed between 15 °C and 75 °C at 0.5 °C/min.

CH_2-PSM/PSM mixture (28.5 °C) was also lower than for both CH_2-PSM and PSM in their pure states, indicating effects on molecular rearrangements in the bilayers. The temperatures of the main transition for the binary mixtures were intermediate between the melting temperatures for the respective pure lipids. The enthalpy for the main transition of NH-PSM/PSM mixture was somewhat higher than for pure NH-PSM suggesting that PSM increased intermolecular interactions, S-PSM and CH_2-PSM had similar main transition enthalpies as in their respective pure states.

3.4. Formations of cholesterol-rich ordered domains investigated through fluorescence quenching of CTL in multilamellar bilayer vesicles

To look at the thermostability of cholesterol-rich ordered domains, in otherwise fluid membranes, the fluorescence quenching of the cholesterol analogue CTL was measured as a function of temperature. CTL was used as a probe for cholesterol-rich ordered domains and the quencher used was 7SLPC, which resides in cholesterol poor disordered domains [27,32]. The F-samples consisted of POPC/7SLPC/PSM-analogue/cholesterol (30:30:30:10, mol%) and the quencher free F_0 sample consisted of POPC/PSM-analogue/cholesterol, (60:30:10, mol%). The F/F_0 ratio shows the relative amount of unquenched fluorescence. The melting of cholesterol-rich ordered domains can be seen as a decrease of the F/F_0 ratio since an increasing amount of CTL is quenched by 7SLPC. Fig. 5 shows the melting of cholesterol-rich ordered domains containing each of the PSM-analogues as a function of temperature. All of the PSM-analogues formed cholesterol-rich ordered domains but with different thermostability. The melting of cholesterol-rich ordered domains containing PSM was complete at approximately 41 °C. Melting of cholesterol-rich ordered domains with S-PSM was complete at approximately the same temperature as PSM whereas the melting of domains with NH-PSM and CH_2-PSM was complete at lower temperatures as compared to domains with PSM.

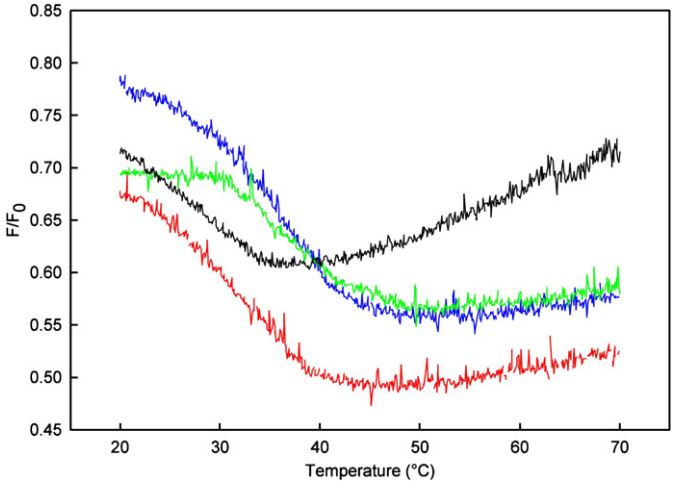


Fig. 5. Fluorescence quenching of CTL with 7SLPC in multilamellar bilayer vesicles. The domain forming lipid in the samples was PSM (blue), NH-PSM (red) S-PSM (green) or CH_2-PSM (black). The total lipid concentration was 50 μM and the graphs are representatives of several independent experiments.

4. Discussion

In this study we have investigated the membrane properties and interactions of proximal esteric oxygen-modified synthetic SMs. Previously, studies in which the effects of structural alterations of SMs on membrane properties have been performed, but none of these have focused on the importance of the oxygens in the phosphocholine linkage [5,10,16,33,34]. We suggest that the modifications of the proximal esteric oxygen are likely to affect the spatial arrangement of the phosphocholine head group, hydrogen bonding and electrostatic interactions of the PSM-analogues.

The hydration of the interface was clearly affected by the modifications in the PSM-analogues. The interfacial hydration for PSM and the PSM-analogues measured with Laurdan GP experiments inversely correlated with the thermal stability of the compounds measured with DSC. The thermal stability had the following order, S-PSM>PSM>NH-PSM>CH_2-PSM, S-PSM with the highest T_m also had the lowest interfacial hydration. The hydration of lipid interfaces is affected by polar group steric factors, electrostatic forces and properties of the hydrophobic part of the molecules [35]. PSM is known to be more hydrated than acyl chain-matched PCPE due to the additional methyl groups [16]. The increased interfacial hydration of NH- and CH_2-PSM could be an effect of direct hydration of the NH- and CH_2-groups or an effect on the orientation of the phosphocholine head group, possibly leading to increased penetration of water into the interfacial region. S-PSM bilayers have an increased order and higher T_m as compared to PSM bilayers, indicating increased intermolecular interactions between the S-PSM molecules. This increase in interactions could also decrease the hydration of the bilayer due to closer contact between the S-PSM molecules. The possibility of differences in

the positioning of Laurdan in bilayers of the compounds examined must also be considered. The molecular differences might affect the environment surrounding Laurdan and thus give rise to the differences in polarity sensed by the Laurdan molecule.

Membrane chain melting is, apart from hydrocarbon chain properties (e.g., unsaturation and length) and aqueous solute properties, affected by the interfacial and head group properties including surface polarity, head group size, interface hydration and intermolecular hydrogen bonding possibilities [36–39]. For SMs also intramolecular hydrogen bonding has been implicated in stabilizing SM/SM interactions [1,2]. The DSC and anisotropy experiments showed that the chain melting temperature of the PSM-analogues was affected by the modifications. The S-linkage increased and the NH- and CH_2-linkages decreased the chain melting temperature as compared to PSM. The size of head groups has been shown to affect interactions between hydrocarbon chains in membrane lipids, since a large head group decreases the interactions between the hydrocarbon chains and lowers the chain melting temperature [40,41]. The head-group size-effect on the PSM-analogue interactions is probably very small, since the size differences between the modifications and the O-linkage in PSM are small. The intramolecular hydrogen bond between the hydroxyl group and phosphate oxygens in SM has been shown to affect the orientation of the phosphocholine head group [3]. Disrupting the hydrogen bond between the proximal esteric oxygen and the hydroxyl group in SM with NH and CH_2-linkages could affect the tilt of the phosphocholine head group and affect PSM-analogue intermolecular interactions. The decrease in chain melting temperature for NH-PSM and CH_2-PSM could also be due to effects on the electrostatic properties of SMs. The partial negative charge of the oxygens in the phosphocholine linkage has been implicated to be important in intermolecular charge-pairing in dimyristoylphosphatidylcholine interactions [42]. The modifications in NH-PSM and CH_2-PSM are likely to affect the charge distribution and intermolecular charge-pairing in SM bilayers.

For S-PSM, with similar orbital arrangements in the S-linkage as PSM has in the O-linkage [20], the disruption of the intramolecular hydrogen bond in SM could make the hydroxyl group of S-PSM available for intermolecular hydrogen bonding. Together with the change in head group orientation this could increase the possibility for intermolecular hydrogen bonding in S-PSM bilayers. Similar increased hydrogen bonding due to closer molecular contact has previously been proposed for DHSM. DHSM, lacking the *trans* double bond in the ceramide backbone, has a more ordered bilayer as compared to PSM [6]. The ordering effect of DHSM is due to increased contact between the DHSM molecules possibly leading to increased intermolecular hydrogen bonding [2,6,7].

The complex melting behavior for NH-PSM and CH_2-PSM indicates that rather large rearrangements of the head groups take place during the transition. S-PSM has a very complex main transition indicating a multi-state transition process. Uncooperative and complex main transitions have earlier been observed for synthetic phosphatidylcholines containing sulfur in thioester linkages and for dipalmitoylthiophosphatidylcholines [43,44]. The lack of a pretransition for S-PSM suggests that the S-linkage possibly affected the head group movement and orientation by making thermal molecular rearrangements unnecessary. It has previously been shown that certain SM-species can form a ripple phase and the pretransitions observed for NH-PSM and CH_2-PSM could indicate the formation of a ripple phase [45,46]. The modifications in the PSM-analogues affected the thermal molecular arrangements in the bilayer vesicles but we cannot however be certain about the nature of the pretransitions and the phase that is formed.

The modifications in the PSM-analogues clearly affected the interactions with cholesterol. Cholesterol has been shown to preferentially interact with SM as compared to acyl chain-matched phosphatidylcholines [47–49]. Cholesterol and sphingolipids have also been shown to be the major components of lipid rafts in cell membranes [50]. Using computational simulations it has been suggested that the observed strong interactions between SM and cholesterol are due to hydrogen bonding, charge-pairing, water shielding, van der Waals interactions and hydrophobic interactions [4,14]. The results from the fluorescence quenching experiments suggested that all analogues formed cholesterol-rich ordered domains but with different thermostability. NH-PSM and CH_2-PSM formed more unstable cholesterol-rich ordered domains as compared to PSM whereas S-PSM formed cholesterol-rich ordered domains with similar thermostability as PSM. The increased hydration of the interface observed for NH- and CH_2-PSM could lead to weaker interactions with cholesterol [17]. The phosphocholine head group has been implicated to shield the cholesterol molecule from interactions with water. Disruption of the intramolecular hydrogen bond between the proximal esteric oxygen and the hydroxyl group of SM could change the orientation of the head group and affect the shielding of cholesterol.

In conclusion, this study has examined the importance of the proximal esteric oxygen in SM/SM and SM/cholesterol interactions and we speculate that the modifications of the proximal esteric oxygen affect the orientation, hydrogen bonding and electrostatic properties of the phosphocholine head group. The S-linkage stabilized intermolecular interactions whereas the NH- and CH_2-linkages destabilized intermolecular interactions in one-component bilayer vesicles. Although all PSM-analogues interacted with cholesterol, the mode of interaction was affected by the head group linkage.

Acknowledgements

We thank Drs Thomas Nyholm, Bodil Ramstedt and Jussi Meriluoto for their valuable comments and help. This project was supported by generous grants from the Academy of Finland, the Sigrid Juselius Foundation, the Waldemar von Frenckell Foundation and the Åbo Akademi Foundation.

References

[1] K.S. Bruzik, Conformation of the polar headgroup of sphingomyelin and its analogues, Biochim. Biophys. Acta 939 (1988) 315–326.
[2] C.M. Talbott, I. Vorobyov, D. Borchman, K.G. Taylor, D.B. DuPre, M.C. Yappert, Conformational studies of sphingolipids by NMR spectroscopy. II. Sphingomyelin, Biochim. Biophys. Acta 1467 (2000) 326–337.
[3] P. Niemela, M.T. Hyvonen, I. Vattulainen, Structure and dynamics of sphingomyelin bilayer: insight gained through systematic comparison to phosphatidylcholine, Biophys. J. 87 (2004) 2976–2989.
[4] J. Aittoniemi, P.S. Niemela, M.T. Hyvonen, M. Karttunen, I. Vattulainen, Insight into the putative specific interactions between cholesterol, sphingomyelin, and palmitoyl-oleoyl phosphatidylcholine, Biophys. J. 92 (2007) 1125–1137.
[5] M. Kuikka, B. Ramstedt, H. Ohvo-Rekila, J. Tuuf, J.P. Slotte, Membrane properties of D-*erythro*-*N*-acyl sphingomyelins and their corresponding dihydro species, Biophys. J. 80 (2001) 2327–2337.
[6] T. Nyholm, M. Nylund, A. Soderholm, J.P. Slotte, Properties of palmitoyl phosphatidylcholine, sphingomyelin, and dihydrosphingomyelin bilayer membranes as reported by different fluorescent reporter molecules, Biophys. J. 84 (2003) 987–997.
[7] T.K. Nyholm, M. Nylund, J.P. Slotte, A calorimetric study of binary mixtures of dihydrosphingomyelin and sterols, sphingomyelin, or phosphatidylcholine, Biophys. J. 84 (2003) 3138–3146.
[8] P.F. Almeida, A. Pokorny, A. Hinderliter, Thermodynamics of membrane domains, Biochim. Biophys. Acta 1720 (2005) 1–13.
[9] B. Ramstedt, J.P. Slotte, Sphingolipids and the formation of sterol-enriched ordered membrane domains, Biochim. Biophys. Acta 1758 (2006) 1945–1956.
[10] R. Bittman, C.R. Kasireddy, P. Mattjus, J.P. Slotte, Interaction of cholesterol with sphingomyelin in monolayers and vesicles, Biochemistry 33 (1994) 11776–11781.
[11] M.P. Veiga, J.L. Arrondo, F.M. Goni, A. Alonso, D. Marsh, Interaction of cholesterol with sphingomyelin in mixed membranes containing phosphatidylcholine, studied by spin-label ESR and IR spectroscopies. A possible stabilization of gel-phase sphingolipid domains by cholesterol, Biochemistry 40 (2001) 2614–2622.
[12] W. Guo, V. Kurze, T. Huber, N.H. Afdhal, K. Beyer, J.A. Hamilton, A solid-state NMR study of phospholipid-cholesterol interactions: sphingomyelin-cholesterol binary systems, Biophys. J. 83 (2002) 1465–1478.
[13] G.A. Khelashvili, H.L. Scott, Combined Monte Carlo and molecular dynamics simulation of hydrated 18:0 sphingomyelin-cholesterol lipid bilayers, J. Chem. Phys. 120 (2004) 9841–9847.
[14] T. Rog, M. Pasenkiewicz-Gierula, Cholesterol-sphingomyelin interactions: a molecular dynamics simulation study, Biophys. J. 91 (2006) 3756–3767.

[15] J. Huang, J.T. Buboltz, G.W. Feigenson, Maximum solubility of cholesterol in phosphatidylcholine and phosphatidylethanolamine bilayers, Biochim. Biophys. Acta 1417 (1999) 89–100.
[16] B. Terova, R. Heczko, J.P. Slotte, On the importance of the phosphocholine methyl groups for sphingomyelin/cholesterol interactions in membranes: a study with ceramide phosphoethanolamine, Biophys. J. 88 (2005) 2661–2669.
[17] J. Huang, G.W. Feigenson, A microscopic interaction model of maximum solubility of cholesterol in lipid bilayers, Biophys. J. 76 (1999) 2142–2157.
[18] T. Hakogi, Y. Monden, M. Taichi, S. Iwama, S. Fujii, K. Ikeda, S. Katsumura, Synthesis of sphingomyelin carbon analogues as sphingomyelinase inhibitors, J. Org. Chem. 67 (2002) 4839–4846.
[19] T. Hakogi, M. Taichi, S. Katsumura, Synthesis of a nitrogen analogue of sphingomyelin as a sphingomyelinase inhibitor, Org. Lett. 5 (2003) 2801–2804.
[20] T. Hakogi, S. Fujii, M. Morita, K. Ikeda, S. Katsumura, Synthesis of sphingomyelin sulfur analogue and its behavior toward sphingomyelinase, Bioorg. Med. Chem. Lett. 15 (2005) 2141–2144.
[21] A. Bjorkbom, T. Yamamoto, S. Kaji, S. Harada, S. Katsumura, J.P. Slotte, Properties of phosphonate oxygen-modified synthetic sphingomyelins and their interactions with cholesterol in monolayers and bilayer membranes, Chem. Phys. Lipids 149 (supplement 1) (2007) S25.
[22] R.T. Fischer, F.A. Stephenson, A. Shafiee, F. Schroeder, delta 5,7,9(11)-Cholestatrien-3 beta-ol: a fluorescent cholesterol analogue, Chem. Phys. Lipids 36 (1984) 1–14.
[23] R. Bittman, Chemical preparation of glycerolipids: a review of recent syntheses, in: G. Cevec (Ed.), Phospholipids handbook, Marcel Dekker, INC., 1993, pp. 154–155.
[24] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Springer Science+Business Media, LLC, New York, 2006.
[25] T. Parasassi, S.G. De, A. d'Ubaldo, E. Gratton, Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence, Biophys. J. 57 (1990) 1179–1186.
[26] S.M. Alanko, K.K. Halling, S. Maunula, J.P. Slotte, B. Ramstedt, Displacement of sterols from sterol/sphingomyelin domains in fluid bilayer membranes by competing molecules, Biochim. Biophys. Acta 1715 (2005) 111–121.
[27] Y.J. Bjorkqvist, T.K. Nyholm, J.P. Slotte, B. Ramstedt, Domain formation and stability in complex lipid bilayers as reported by cholestatrienol, Biophys. J. 88 (2005) 4054–4063.
[28] R.D. Kaiser, E. London, Location of diphenylhexatriene (DPH) and its derivatives within membranes: comparison of different fluorescence quenching analyses of membrane depth, Biochemistry 37 (1998) 8180–8190.
[29] P.L. Chong, P.T. Wong, Interactions of Laurdan with phosphatidylcholine liposomes: a high pressure FTIR study, Biochim. Biophys. Acta 1149 (1993) 260–266.
[30] T. Parasassi, E.K. Krasnowska, L. Bagatolli, E. Gratton, Laurdan and Prodan as polarity-sensitive fluorescent membrane probes, J. Fluoresc. 8 (1998) 365–373.
[31] T. Parasassi, S.G. De, G. Ravagnan, R.M. Rusch, E. Gratton, Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence, Biophys. J. 60 (1991) 179–189.
[32] S.N. Ahmed, D.A. Brown, E. London, On the origin of sphingolipid/cholesterol-rich detergent-insoluble cell membranes: physiological concentrations of cholesterol and sphingolipid induce formation of a detergent-insoluble, liquid-ordered lipid phase in model membranes, Biochemistry 36 (1997) 10944–10953.
[33] L. Gronberg, Z.S. Ruan, R. Bittman, J.P. Slotte, Interaction of cholesterol with synthetic sphingomyelin derivatives in mixed monolayers, Biochemistry 30 (1991) 10746–10754.
[34] C.C. Kan, Z.S. Ruan, R. Bittman, Interaction of cholesterol with sphingomyelin in bilayer membranes: evidence that the hydroxy group of sphingomyelin does not modulate the rate of cholesterol exchange between vesicles, Biochemistry 30 (1991) 7759–7766.
[35] R.P. Rand, V.A. Parsegian, The forces between interacting bilayer membranes and the hydration of phospholipid assemblies, in: P.L. Yeagle (Ed.), The structure of biological membranes, CRC press LLC, Boca Raton, 2005, pp. 201–242.
[36] A. Blume, H. Eibl, The influence of charge on bilayer membranes. Calorimetric investigations of phosphatidic acid bilayers, Biochim. Biophys. Acta 558 (1979) 13–21.
[37] J.M. Boggs, Lipid intermolecular hydrogen bonding: influence on structural organization and membrane function, Biochim. Biophys. Acta 906 (1987) 353–404.
[38] G. Cevc, How membrane chain-melting phase-transition temperature is affected by the lipid chain asymmetry and degree of unsaturation: an effective chain-length model, Biochemistry 30 (1991) 7186–7193.
[39] G. Cevc, How membrane chain melting properties are regulated by the polar surface of the lipid bilayer, Biochemistry 26 (1987) 6305–6310.
[40] H.J. Hinz, L. Six, K.P. Ruess, M. Lieflander, Head-group contributions to bilayer stability: monolayer and calorimetric studies on synthetic, stereochemically uniform glucolipids, Biochemistry 24 (1985) 806–813.
[41] K. Iwamoto, J. Sunamoto, K. Inoue, T. Endo, S. Nojima, Liposomal membranes. XV Importance of surface structure in liposomal membranes of glyceroglycolipids, Biochim. Biophys. Acta 691 (1982) 44–51.
[42] M. Pasenkiewicz-Gierula, Y. Takaoka, H. Miyagawa, K. Kitamura, A. Kusumi, Charge pairing of headgroups in phosphatidylcholine membranes: a molecular dynamics simulation study, Biophys. J. 76 (1999) 1228–1240.
[43] J. Hajdu, J.M. Sturtevant, Phospholipids showing complex bilayer phase transitions. II. Four sulfur-containing phosphatidylcholines, Chem. Phys. Lipids 55 (1990) 323–330.
[44] D.A. Wisner, T. Rosariojansen, M.D. Tsai, Phospholipids chiral at phosphorus. 12. configurational effect on the thermotropic properties of chiral dipalmitoylthio-phosphatidylcholine, J. Am. Chem. Soc. 108 (1986) 8064–8068.
[45] H.W. Meyer, H. Bunjes, A.S. Ulrich, Morphological transitions of brain sphingomyelin are determined by the hydration protocol: ripples re-arrange in plane, and sponge-like networks disintegrate into small vesicles, Chem. Phys. Lipids 99 (1999) 111–123.
[46] H. Takahashi, T. Hayakawa, Y. Kawasaki, K. Ito, T. Fujisawa, M. Kodama, T. Kobayashi, Structural characterization of *N*-lignoceroyl (C24: 0) sphingomyelin bilayer membranes: a re-evaluation, J. Appl. Crystallogr. 40 (2007) S312–S317.
[47] R.A. Demel, J.W. Jansen, P.W. van Dijck, L.L. van Deenen, The preferential interaction of cholesterol with different classes of phospholipids, Biochim. Biophys. Acta 465 (1977) 1–10.
[48] P. Mattjus, J.P. Slotte, Does cholesterol discriminate between sphingomyelin and phosphatidylcholine in mixed monolayers containing both phospholipids? Chem. Phys. Lipids 81 (1996) 69–80.
[49] B. Ramstedt, J.P. Slotte, Interaction of cholesterol with sphingomyelins and acyl-chain-matched phosphatidylcholines: a comparative study of the effect of the chain length, Biophys. J. 76 (1999) 908–915.
[50] K. Simons, E. Ikonen, Functional rafts in cell membranes, Nature 387 (1997) 569–572.